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14. ABSTRACT Prostate cancer (PCa) remains the most common malignancy and the second leading cause of cancer-related death for men in the United States. Recent studies have shown significant interactions between nerves and adjacent cancer cells that promote cell survival, proliferation and migration of PCa cells. Our studies of laser captured prostate cancer reactive stroma have shown that among the most upregulated genes is glial cell line-derived neurotrophic factor (GDNF), which is expressed by peripheral nerves. GDNF binds to RET, a receptor tyrosine kinase, in conjunction with its co-receptor GFR α 1 (GFRA1) and activates cellular signaling. Both RET and GFRA1 are expressed on all PCa cell lines tested and RET protein is increased in PCa. Studies in pancreatic cancer strongly implicate GDNF as a key factor promoting perineural migration. We will test the hypothesis that GDNF is expressed by nerves and that it acts on RET/GFRA1 in adjacent PCa cells to promote proliferation and invasion and inhibit apoptosis and that disruption of this signaling cascade will inhibit PCa progression in vivo.					
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1. INTRODUCTION.

Prostate cancer (PCa) remains the most common malignancy and the second leading cause of cancer-related death for men in the United States. The tumor microenvironment plays an important role in the initiation and progression of PCa. One important component of this microenvironment is nerves. PCa has a propensity to grow in perineural locations and large diameter perineural tumor is one of the most significant pathological predictors of poor outcome following radical prostatectomy. Perineural invasion is also associated with poor outcomes following radiation therapy. Recent studies have shown significant interactions between nerves and adjacent cancer cells that promote cell survival, proliferation and migration of PCa cells. Recent landmark studies have shown that chemical or surgical ablation of nerves inhibits tumorigenesis and metastasis in both xenograft and transgenic mouse models of PCa, unequivocally establishing that nerve-PCa cell interactions play a significant role in PCa initiation and progression. Interestingly, PCa cells and nerves have bidirectional interactions. Recent studies have shown that PCa induces axonogenesis and neurogenesis and higher nerve densities in the microenvironment are associated with aggressive clinical behavior in PCa. **Thus nerves and PCa cells interact in a potential feed forward loop in which nerves promote prostate cancer cell proliferation, survival and invasion and PCa cells in turn increase the number of nerve fibers via increased axonogenesis and neurogenesis.**

Our studies of laser captured prostate cancer reactive stroma¹ have shown that among the most upregulated genes is glial cell line-derived neurotrophic factor (GDNF), which is expressed by peripheral nerves. GDNF binds to RET, a receptor tyrosine kinase, in conjunction with its co-receptor GFR α 1 and activates cellular signaling. Studies in pancreatic cancer strongly implicate RET signaling as a key factor promoting perineural migration. Our goal is to evaluate the role of RET signaling in the response of PCa to adjacent nerves and to determine if disruption of this signaling cascade will inhibit PCa progression in vivo.

2. KEYWORDS: prostate cancer; nerve, RET, GDNF, GFR α 1

3. OVERALL PROJECT SUMMARY:

Major Task 1. Obtain regulatory approvals for animal and human protocols (Months 1-3)

All regulatory approvals have been obtained and maintained.

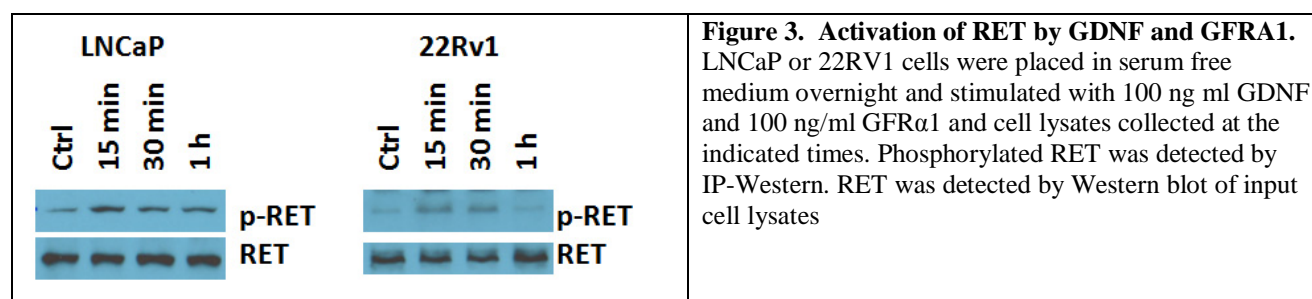
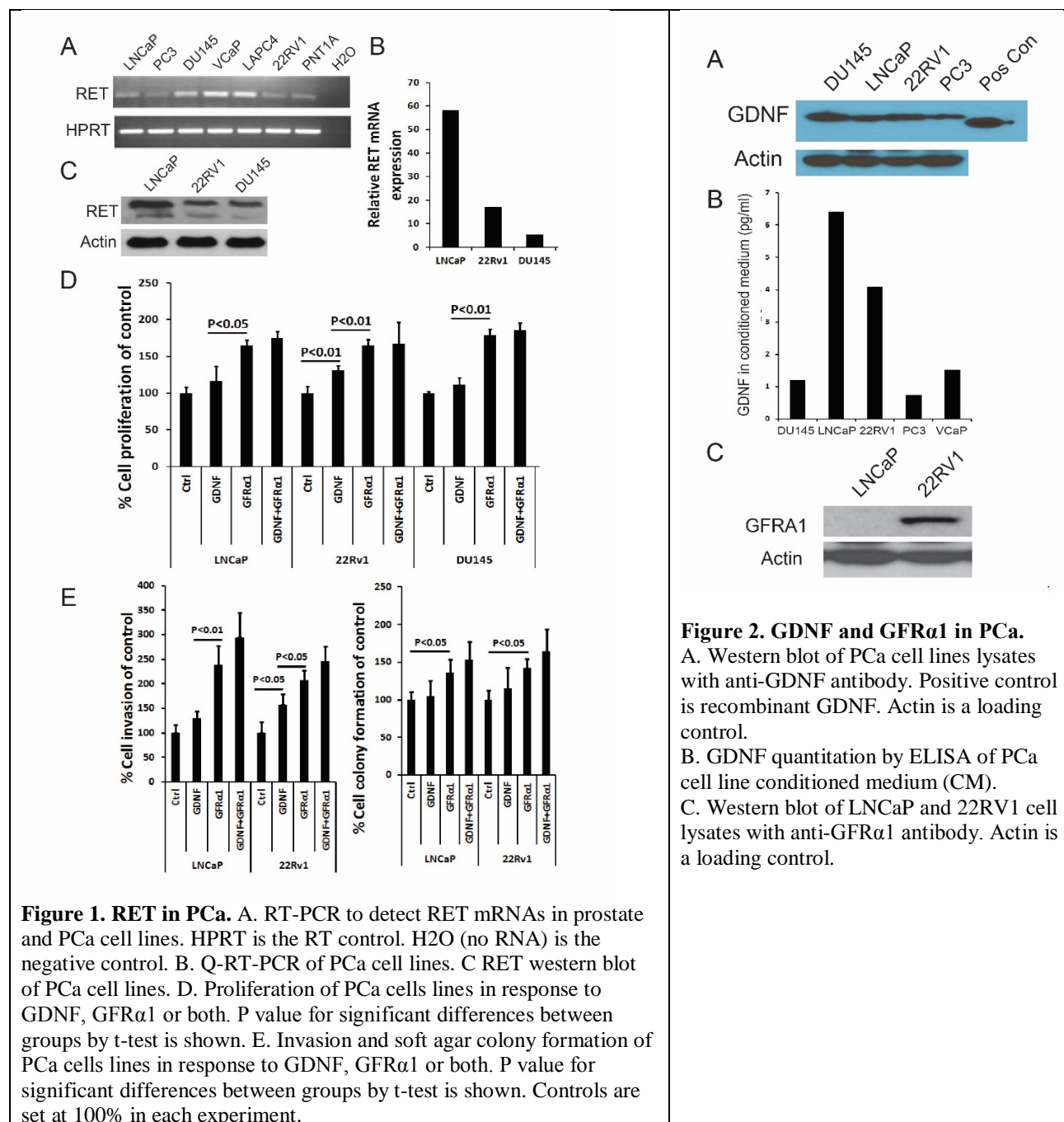
Major Task 2: In vitro studies of GDNF.

Subtask 1: Sub-aim 1.1. Phenotypic effects of GDNF on prostate cancer cells in vitro (months 1-12).

Given that GDNF is secreted in the tumor microenvironment we initially sought to determine if RET is expressed in a broad array of PCa cells. RT-PCR of RNAs from all PCa cell lines and the immortalized prostate epithelial cell line PNT1a shows that all PCa cell lines and prostate epithelial cells express RET mRNA at variable levels (Fig 1A). Western blot analysis confirmed RET expression at the protein level that was concordant with mRNA levels measured by Q-RT-PCR (Fig 1B and 1C). Both GDNF and GFR α 1 are released by nerves and have been previously shown to be able to induce cancer cell proliferation and invasion. We therefore treated PCa cell lines with GDNF, GFR α 1 or both and evaluated proliferation (Fig 1D). Somewhat surprisingly, all cell lines responded to GFR α 1 alone but only 22RV1 cells responded to GDNF alone. Furthermore, combined treatment with GDNF and GFR α 1 was no more potent than GFR α 1 alone. Similar results were seen when invasion and soft agar colony formation were assessed (Fig 1E). These findings implied that GDNF was present in the PCa cell lines since all cell lines responded to exogenous GFR α 1. Furthermore, only 22RV1 cells can respond to exogenous GDNF, implying they are the only PCa cell line that expresses GFR α 1. Indeed, as seen in Fig 2A, by western blotting and ELISA for GDNF, all PCa cell lines expressed variable levels of GDNF protein. This indicates that GDNF can act as an autocrine factor in PCa, in addition to its role as a potential paracrine factor from nerves. Furthermore, RT-PCR analysis showed that only 22RV1 expressed significant amounts of GFR α 1 and western blot shows that 22RV1 are the only PCa cell lines to express GFR α 1 protein (Fig 2C). It should be noted that even 22RV1 responded to exogenous GFR α 1, indicating that exogenous GFR α 1 protein can enhance the response of PCa cells to GDNF even in the presence of endogenous GFR α 1. Interestingly, new data has emerged from the pancreatic cancer literature relevant to our observations. He et al² have recently reported that soluble GFR α 1 is released by nerves and plays a key role in enhancing nerve cancer interactions. We believe that a similar situation applies to PCa.

Subtask 2: Sub-aim 1.2. Pathway activation by GDNF

We have undertaken a series of experiments using western blotting and reverse phase protein arrays to examine pathway activation in PCa cells after activation of RET signaling. As shown in Figure 3, RET signaling is activated with rapid kinetics after stimulation of PCa cell lines with GDNF and GFR α 1. RET phosphorylation increased within 15 minutes after stimulation.



A

LNCaP				22Rv1				
Ctrl	GDNF	GFRα1	GDNF+GFR α1	Ctrl	GDNF	GFRα1	GDNF+GFR α1	
								p-AKT
								AKT
								p-ERK1/2
								ERK1/2
								p-p70S6K
								p70S6K
								β-Actin

B

LNCaP			22Rv1			
Ctrl	LY294002	U0126	Ctrl	LY294002	U0126	
						p-AKT
						AKT
						p-ERK1/2
						ERK1/2
						p-p70S6K
						p70S6K
						β-Actin

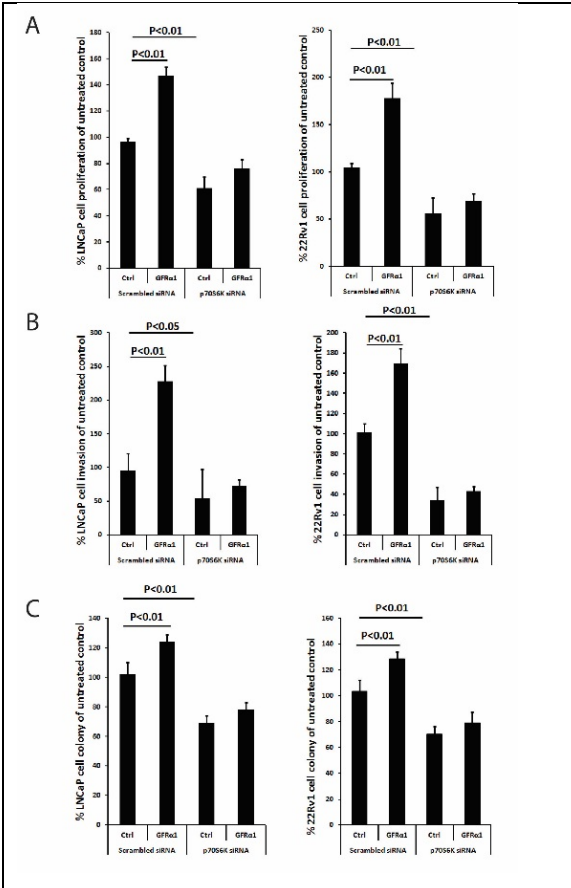
C

LNCaP				22Rv1				
Scrambled siRNA	Scrambled siRNA	RET siRNA	RET siRNA	Scrambled siRNA	Scrambled siRNA	RET siRNA	RET siRNA	
								p-AKT
								AKT
								p-ERK1/2
								ERK1/2
								p-p70S6K
								p70S6K
								β-Actin

GFRα1 - + - + - + - +

Figure 4 (left). Signaling events induced by treatment of PCa cell lines with GDNF, GFRα1 or both.
A. Western blots of indicated proteins or phosphoproteins are shown. B. Experiment is similar to A except cells were treated with the PI3-K inhibitor LY294002 or the MAPK inhibitor U0126
U0126

Figure 5 (above). Knockdown of RET inhibits activation of pathways by GFRα1. PCa cells were treated with siRNA targeting RET or controls and then stimulated with GFRα1. Western blots of indicated proteins or phosphoproteins are shown.



Transient p70S6K siRNA transfections into LNCaP and 22RV1 cells were used to evaluate the role of p70S6K in the effect of GFRα1 on the biological alterations induced by GFRα1. The results show that knockdown of p70S6K markedly reduced cell proliferation (Fig 6A), invasion (Fig 6B) and colony formation (Fig 6C) in response to GFRα1.

Figure 6 (left). Knockdown of p70S6K markedly inhibits GFRα1 induced phenotypes. Cells were pretreated with siRNA targeting p70S6K of control siRNA and treated with GFRα1 and proliferation, invasion or soft agar colony formation measured. P values for significant differences by t-test are shown.

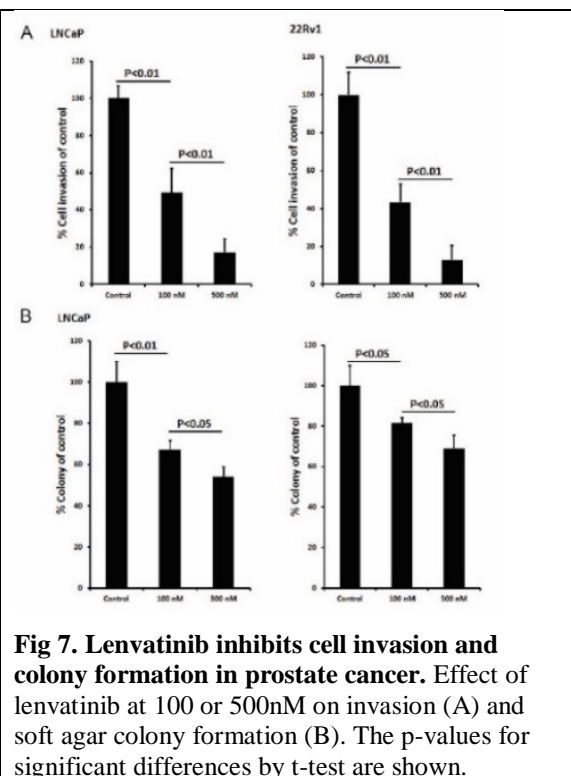


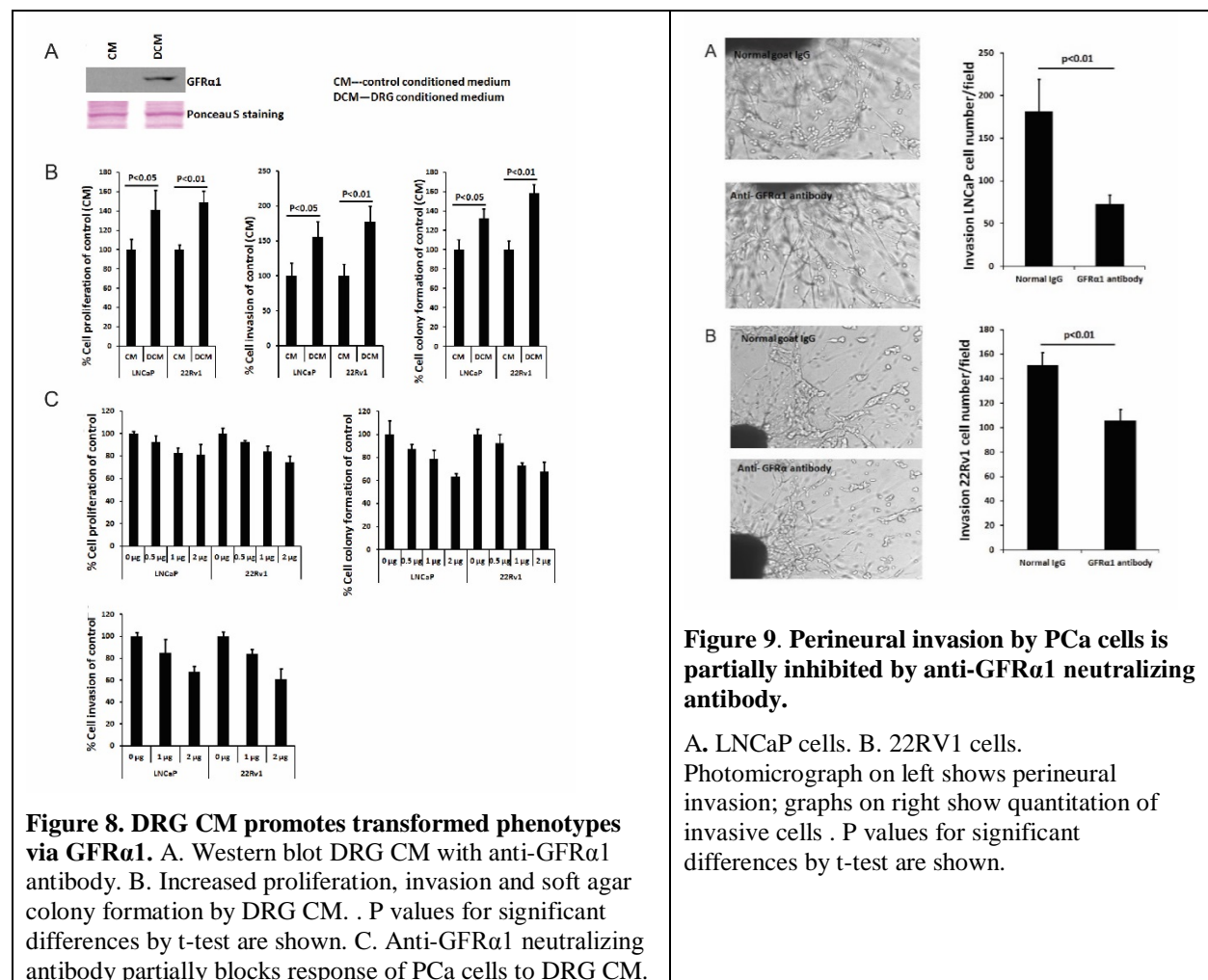
Fig 7. Lenvatinib inhibits cell invasion and colony formation in prostate cancer. Effect of lenvatinib at 100 or 500nM on invasion (A) and soft agar colony formation (B). The p-values for significant differences by t-test are shown.

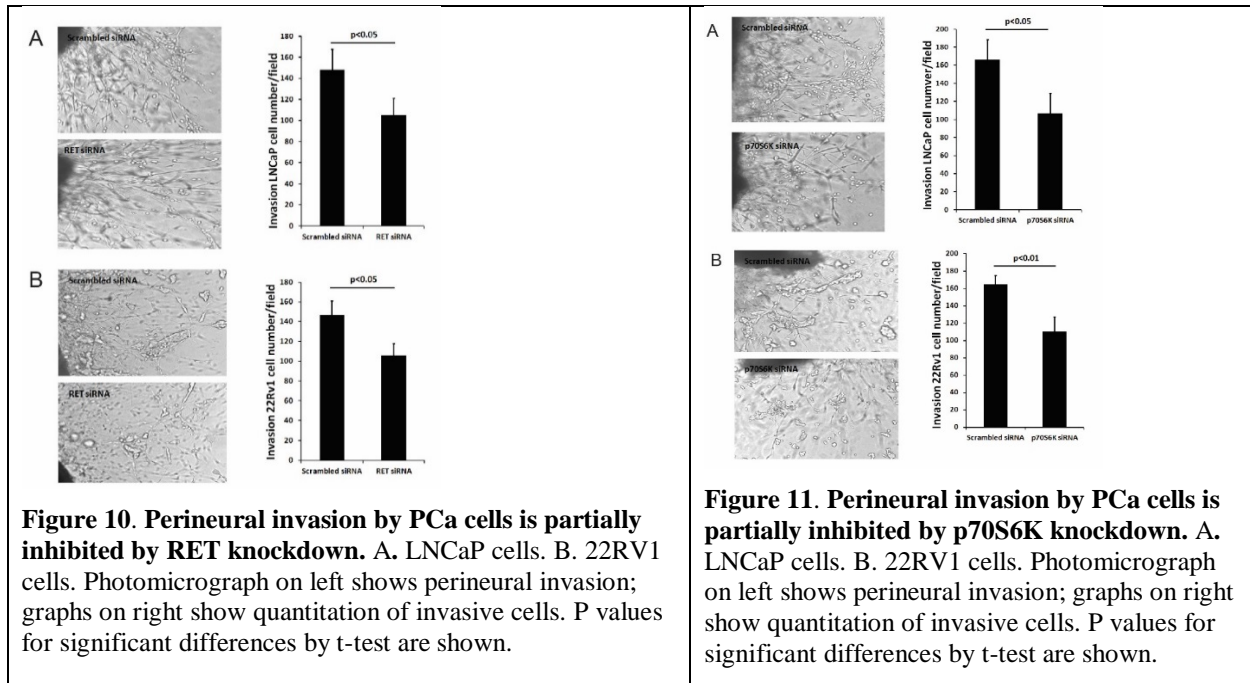
Our data supports an important role RET in PCa. This is clinically relevant since RET signaling can be inhibited by lenvatinib, a multikinase inhibitor that inhibits RET, FGFRs, VEGFRs and PDGFRs³. This drug is in the clinic and approved for treatment of advanced thyroid and renal cancer. We have found that lenvatinib strongly inhibits invasion and colony formation in both LNCaP and 22RV1 cells (Fig 7). If our hypothesis is correct, treatment of men with lenvatinib may enhance long term treatment outcomes in men with more aggressive disease.

Major Task 3: Role of GDNF in nerve-prostate cancer cell interactions.

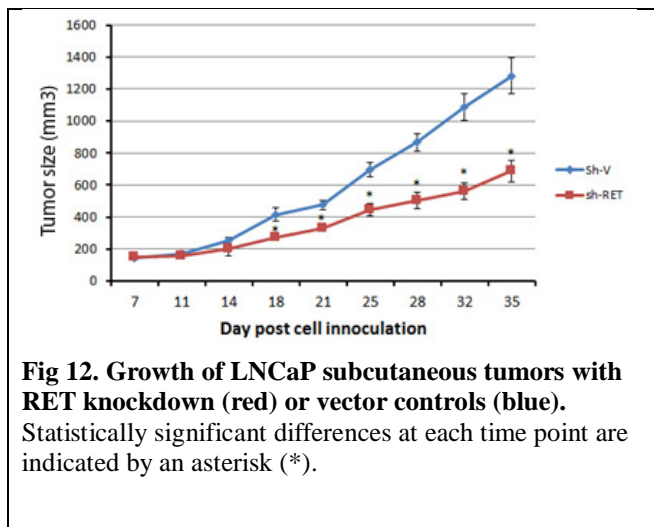
It is known that nerves can release soluble GFR α 1. We therefore examined the ability of dorsal root ganglia (DRG) excised from mice to enhance cancer phenotypes in PCa cells in vitro. As expected, GFR α 1 is present in the conditioned media (CM) of DRG in cultures (Fig 8A). CM from DRG was able to significantly enhance proliferation, invasion and soft agar colony formation in both LNCaP and 22RV1 cells (Fig 8B). This was blocked (although not completely) by neutralizing anti-GFR α 1 antibody (Fig 8C).

Using the DRG in vitro coculture model of perineural invasion, we found that LNCaP and 22RV1 cancer cells invaded along the neurites and that perineural invasion was significantly attenuated by anti-GFR α 1 antibody (Fig 9), suggesting that GFR α 1 also plays a critical role in the perineural invasion. Knockdown of RET with siRNA also markedly inhibited perineural invasion (Fig 10). Moreover, knockdown of p70S6K also decreased perineural invasion (Fig.11). These findings suggest that p70S6K plays a pivotal role in the effect of GFR α 1/RET on these cellular behaviors.

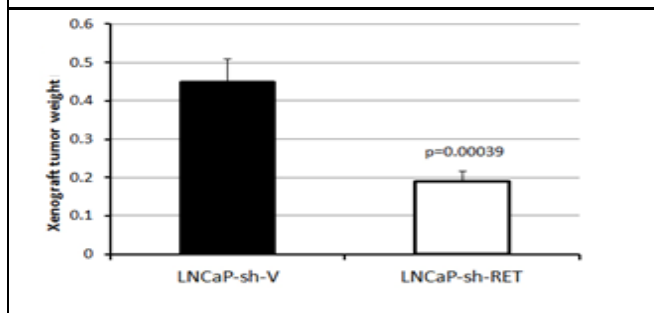




Subtask 3: Sub-aim 2.3. Role of RET in prostate cancer progression in vivo (months 4-36)



To determine whether RET signaling promotes tumor growth in vivo we established LNCaP cells with RET knockdown and vector controls. We then evaluated tumor growth in vivo using a subcutaneous xenograft model. As shown in Figures 12 and 13, cells with RET knockdown grew significantly more slowly than vector controls (Fig 12), with lower final tumor weights (Fig 13).

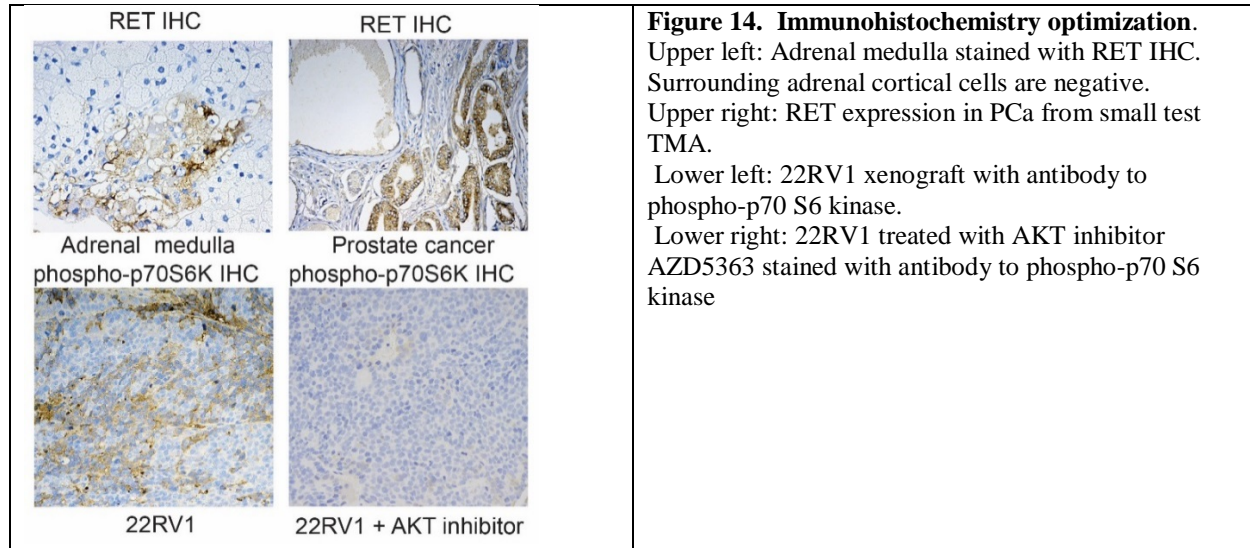


Mean +/- SEM of tumor weight (in gms) is shown. P value by t-test was highly significant and is shown

Mean +/- SEM of tumor weight (in gms) is shown. P value by t-test was highly significant and is shown

Major Task 3: In vivo expression of RET, GRF α 1 and GDNF in prostate cancer

We have optimized immunohistochemistry for RET (Fig 14) and stained a large TMA with >300 cases of PCa. Given that p70S6K plays a key role in RET signaling in PCa (see above) we have also developed optimized conditions for detecting phosphorylated p70S6K by IHC (Fig 14)



We then examined the expression of RET in clinically localized prostate adenocarcinomas using a tissue microarray containing 325 evaluable clinically localized cancers from radical prostatectomies that has been described previously⁴ using immunohistochemistry. Positive control tissue was adrenal medulla (Fig 14). Intensity (0-3) and extent (0-3) of staining was scored to derive a multiplicative index ranging from 0-9, with 9 representing strong diffuse staining (Fig 15A) as described previously⁴. A total of 61 cancers (18.8%) showed staining. Some had weak staining (≤ 3 , shown in Fig 15B), but this staining is almost certainly biologically significant since LNCaP xenografts, which are inhibited by RET knockdown, show similar intensity of staining by immunohistochemistry (Fig 15C). Interestingly, staining was highly cancer specific, with only a single case showing staining in benign luminal epithelium ($p < .001$; chi-squared). Nerves and ganglia in the stroma of occasional tissue microarray cores were stained as expected as an internal positive control (Fig 15D). Supporting these findings, examination of TCGA databases in cBioportal show that PCa has the third highest RET mRNA levels after pheochromocytoma/ paraganglioma and breast cancer (Figure 16). Of course, pheochromocytoma and paraganglioma would be expected to express high levels of RET since their tissues of origin intrinsically express high levels of RET. It has also been shown that more than half of all breast cancers express RET by immunohistochemistry⁵.

Recent reports have shown that RET mRNA is expressed in lung small cell carcinoma cell lines⁶ and in small intestinal neuroendocrine tumors⁷. We therefore examined whether RET protein is expressed in small cell neuroendocrine cancers of the prostate, which are highly aggressive tumors that can be present at initial diagnosis or arise from adenocarcinomas after prolonged treatment of with androgen blockade. We examined a radical prostatectomy which contained extensive areas of small cell carcinoma as well as high grade prostate adenocarcinoma. The majority of the adenocarcinoma component was negative for RET protein while the

4. KEY RESEARCH ACCOMPLISHMENTS:

- We have shown that multiple prostate cancer cell lines express RET and respond to RET signaling with increased proliferation, invasion and soft agar colony formation.
- Surprisingly, GDNF is expressed as an autocrine factors by PCa cell lines in addition to being expressed by nerves
- GFR α 1 is only weakly expressed by 22RV1 cells of all PCa cell lines tested and thus RET signaling is dependent on GFR α 1 secreted primarily by nerves to promote RET signaling
- We have shown that GDNF/GFR α 1 activate either ERK and AKT signaling in prostate cancer, which are required to activate the downstream target p70S6 kinase by phosphorylation
- Activation of p70S6K plays a key role in enhancing transformed phenotypes induced by RET signaling
- Lenvatinib, a multikinase inhibitor that inhibits RET, decreases invasion and soft agar colony formation by PCa cells in vitro.
- RET signaling activated by GFR α 1 plays an important role in perineural invasion
- Knockdown of RET significantly inhibits tumor growth in vivo
- We have established optimized immunohistochemistry for RET and phospho-P70S6K
- RET protein is expressed on ~20% of clinically localized prostate cancers based on analysis of a large tissue microarray.
- RET is expressed in neuroendocrine prostate cancers

5. CONCLUSION

Our data show that RET is expressed in PCa and responds to GDNF in the presence of GFR α 1 to promote transformed phenotypes including proliferation, invasion, soft agar colony formation and tumor growth in vivo. Somewhat surprisingly, we have found that GDNF is expressed as an autocrine factor in PCa in addition to its paracrine role as a growth factor secreted by nerves. Furthermore, the presence of GFR α 1, which is derived mainly from nerves, appears to be the limiting factor in RET signaling. Thus, a perineural niche favoring RET signaling is created in PCa. RET signaling enhances both ERK and AKT signaling, both of which activate p70S6 kinase. Our data supports the concept that p70S6 kinase is a key downstream target of RET signaling. Our studies indicate that RET signaling is a potential therapeutic target in PCa. RET inhibitors such as lenvatinib (although not specific) are already in clinical use and thus our studies have potential translational implications.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Publication in Clinical Cancer Research

Ban K, Feng S, Shao L, Ittmann M. RET signaling in prostate cancer. Clin Cancer Res. 2017;23(16):4885-96. PMCID: 5609835.

7. INVENTIONS, PATENTS AND LICENSES: None

8. REPORTABLE OUTCOMES: None

9. OTHER ACHIEVEMENTS: None

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